Naturally acquired microchimerism
Implications for transplantation outcome and novel methodologies for detection

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Microchimerism represents a condition where one individual harbors genetically distinct cell populations, and the chimeric population constitutes <1% of the total number of cells. The most common natural source of microchimerism is pregnancy. The reciprocal cell exchange between a mother and her child often leads to the stable engraftment of hematopoietic and non-hematopoietic stem cells in both parties. Interaction between cells from the mother and those from the child may result in maternal immune cells becoming sensitized to inherited paternal alloantigens of the child, which are not expressed by the mother herself. Vice versa, immune cells of the child may become sensitized toward the non-inherited maternal alloantigens of the mother. The extent of microchimerism, its anatomical location, and the sensitivity of the techniques used for detecting its presence collectively determine whether microchimerism can be detected in an individual. In this review, we focus on the clinical consequences of microchimerism in solid organ and hematopoietic stem cell transplantation, and propose concepts derived from data of epidemiologic studies. Next, we elaborate on the latest molecular methodology, including digital PCR, for determining in a reliable and sensitive way the extent of microchimerism. For the first time, tools have become available to isolate viable chimeric cells from a host background, so that the challenges of establishing the biologic mechanisms and function of these cells may finally be tackled.

History of chimerism

The very first observation of chimerism was reported in 1945 by Ray Owen[1] (reviewed in ref. 2). A cow had given birth to twin calves, which turned out to be derived from two different bulls. This form of fraternal twinning is relatively common in cattle. Furthermore, each of these genetically dissimilar twins carried blood antigens from the mother as well as from both sires. Owen went on to systematically study the blood type in 80 pairs of bovine heterozygotic twin calves, and he found that it was identical between the dissimilar twins in the majority of cases.[1] He attributed this result to vascular anastomosis between the placentas of bovine twins. The individual calves displayed a situation, which is nowadays called "mixed chimerism," where cells from two distinct zygote lineages coexist in one organism. In rare human cases, blood from healthy donors, who have a twin sibling, was found to be a mixture of two kinds of blood cells.[3-5] Studies by Billingham, Brent, Medawar, and Hasek provided support for the principle that mammals and birds immunologically react only to a limited extent to foreign tissue cells to which they have been previously exposed in fetal/neonatal life.[6-8] In neonatal mice artificially-induced "macrochimerism" by blood cell transfusion led to antigen-specific tolerance and the acceptance of a skin graft given later in life.[6] Owen and colleagues later provided evidence for the existence of actively acquired tolerance to rhesus (Rh) blood group antigens: Rh-negative children of Rh-positive mothers acquire persistent tolerance toward the Rh antigen,[9] possibly as a result of exposure to the antigen in the uterus. The concept, that the degree of mother's tolerance toward the child's RhD antigen is related to the RhD status of the grandmother,[9] and the role of chimerism therein, has recently been debated.[10]

Mixed Chimerism (Macrochimerism) as a Result of Hematopoietic Stem Cell Transplantation (HSCT)

The balance between immunity and immune regulatory mechanisms will determine the degree of alloimmune responses induced in transplant recipients, and may thereby have significant clinical implications in relation to overall survival. However, immune regulation does not occur without immunity. This concept also applies to conditions of donor-specific immunologic tolerance. Tolerance to particular antigens probably arises from the fact that the effect of regulatory immune cells dominates

over mechanisms exploited by effector type immune cells. To understand the biologic mechanisms of microchimerism, as discussed below, it is helpful to first evaluate the underlying immune modulatory mechanisms of mixed chimerism (macrochimerism) and their impact on clinical outcome.

Mixed chimerism: the ultimate platform to induce immunological tolerance to solid allografts?

The feasibility of inducing long-lasting donor-specific tolerance through the establishment of mixed or even full donor chimerism has been studied in animal models and in humans.[11-16] This type of immune intervention may form the ultimate clinical platform for obtaining sustained allograft function without the need for life-long immunosuppressive medication. The immunological concept of this approach is simple: together with active removal of pre-existing host T cells by conditioning therapy, infusion of hematopoietic stem cells obtained from the same donor as the one who will donate the kidney allograft introduces new donor-derived antigen-presenting cells (APC) along the host-derived APC that were already present. Both types of APC will, among other locations, end up in the thymus where they facilitate the deletion of high avidity T cells that are specific for donor- or host-specific alloantigens. Consequently, the new peripheral alloreactive T cell pool generated after the HSCT procedure will be grossly devoid of cells that recognize donor-specific human leukocyte antigen (HLA) class I or II molecules expressed on cells present in the subsequently transplanted kidney allograft. This so called deletional tolerance occurs through positive and negative selection of immature host T cells facilitated by thymus-residing APC (central tolerance), as well as through deletion of host alloreactive T cells upon interaction with donor HLA-expressing leukocytes present in the periphery (peripheral tolerance).

The concept outlined above was shown to be robust in small and large animal models.[15,17,18] In the nineties, sporadic clinical cases have been described where immunologic tolerance toward the renal allograft was observed after a bone marrow transplant from the same donor, wherein no or minimal dosing of immunosuppressive medication was applied.[19-21] In this new millennium, the potential applicability of combined HSCT and kidney transplantation procedures has been further explored in the clinic.[13,14,22,23] From seven patients who underwent such combined treatment for multiple myeloma and end-stage renal failure, five are alive with no signs of a relapse of the malignancy, and three out of these five have normal to near-normal renal function in complete absence of immunosuppressive therapy.[23] Although substantial co-morbidities were observed in this very small patient group, sustained mixed chimerism, particularly in the T cell population, is perhaps the most ideal setting to assure protection from allograft rejection by host T cells as well as from graft-vs.-host disease (GvHD) inflicted by donor T cells. Similarly, durable chimerism in transplant recipients was associated with tolerance toward the donor kidney allograft after a mobilized stem cell graft (enriched for hematopoietic stem cells and plasmacytoid precursor dendritic cells) and non-myeloablative conditioning.[24] GvHD was not observed. Presence of sustained chimerism was used as an indicator for successfully weaning off immunosuppressive therapy by one year after transplantation in five transplant recipients.

Persistent mixed chimerism is indicative of the co-presence of host- and donor-derived regulatory T cells

Persistent mixed chimerism is frequently observed in Thalassemia major patients after they have undergone HLA-identical HSCT following myeloablative conditioning. In this setting, the proportion of residual host hematopoiesis may range from 10 to 70%.[25] Although residual (host-derived) APC after HSCT are generally considered as a risk factor for graft rejection, Thalassemia major patients in whom host and donor cells co-persist for more than two years after graft infusion often remain blood transfusion-independent for prolonged periods of time.[26,27] T cell cloning experiments showed that mixed chimerism in these patients is associated with the presence of both effector cells and regulatory T cells (Tregs) reactive against host or donor alloantigens.[27] The Treg clones reciprocally inhibited proliferation and IFNγ release by effector type alloreactive T cells of host and donor origin, respectively. The immunesuppressive function of these Tregs depends, at least in part, on the cytokine IL-10. These observations point out that persistent clinical presentation of mixed chimerism after allogeneic HSCT is likely the result of both central tolerance and peripheral tolerance induced by host- and donor-derived alloreactive Tregs. The next challenge will be to find effective and safe conditioning regimens, which favor the induction of stable mixed chimerism without unwanted side effects, as myeloablative conditioning is often accompanied by toxicity issues that may cause severe transplantation-related morbidity and mortality.

Microchimerism as a Result of Solid Organ Transplantation

Donor microchimerism in the recipient

In contrast to hematopoietic macrochimerism (often >25% as observed by Owen, Medawar, and colleagues, and by the group of Sykes and Sachs), a much lower extent of chimerism, usually less than 0.1%, is frequently seen after solid organ transplantation. Low levels of donor chimerism, referred to as "microchimerism," result from the migration of passenger hematopoietic (stem) cells from the allograft into the peripheral circulation and tissues of the recipient. While macrochimeric conditions appear to be related to tolerance mechanisms, the clinical impact of donor microchimerism in solid organ transplantation is less clear.[28] In several studies it was shown that microchimerism in humans is associated with graft acceptance[29,30] and a lower incidence of rejection[31,32] after kidney, liver or small bowel transplantation. In one of those studies, transplant recipients, who displayed microchimerism at 2 mo after graft kidney implantation, had a significantly lower incidence of biopsy-proven acute rejection during the subsequent four years compared with patients who were microchimerism-negative.[32] Whereas in a mouse model it was shown that microchimerism was the causal factor in maintaining deletion of donor-specific CD8+ T cells,[33]

Table 1. Naturally acquired, circulating, or tissue-resident microchimeric cell types identified in humans and mice

Hematopoietic cell types	References
CD34+ / c-kit+ stem cells	57,58,68
CD66b+ granulocytes	51,86
CD3+ T cells	53,86
CD56+ CD16+ natural killer cells	53
CD20+ B cells	53,86
CD14+ monocytes	53,86
CD11b+ macrophages	68
CD11c+ myeloid dendritic cells	55,86,157
Non-hematopoietic cell types	**References**
CD45- / c-kit- bone marrow mesenchymal stromal cells	60,68
Cardiomyocytes	44,158
Kidney tubular epithelial cells	44
Hepatocytes	44,56
Lung epithelial cells	56
Pancreatic insulin-producing β cells	56,159

microchimerism after human transplantation does not seem to be accompanied by diminished anti-donor T cell responses.[31] In a girl who had received a liver allograft from a deceased, unrelated male donor, spontaneous donor chimerism (94–100%) was observed in T, B, and NK cells, and granulocytes at 13 mo post-transplantation.[34] After reduction and complete withdrawal of all immunosuppressive medication the recipient showed immunological tolerance toward the graft.

However, several clinical studies did not show an effect of microchimerism on acceptance of heart, liver, and kidney grafts.[35–37] Microchimerism after liver transplantation has even been observed coinciding with graft rejection.[38,39] Discrepancies in the clinical impact of microchimerism may be the result of inter-patient differences in the level or subtypes of microchimeric cells. Indeed, the type of microchimeric cell has been advocated to be the underlying factor determining whether microchimerism is related to tolerance or immunity toward host-derived foreign cells.

Recipient chimerism in the donor organ

Recipient chimerism in transplants probably results from migration of precursor- and/or stem cells from the recipient into the transplanted donor organ. Recipient chimerism was observed in endothelial cells of kidney grafts.[40] The highest frequencies of chimerism were seen during the most severe types of rejection, i.e., vascular rejection. In another study, 88% of kidney transplant patients had chimerism in 2.4 to 6.6% of tubular epithelial cells in their donor graft.[41] This tubular chimerism was not related to clinical outcome. Renal allografts undergoing chronic dysfunction often contain mesenchymal cells from recipient origin in vascular and interstitial compartments.[42] In female donor heart transplants, Y-positive chimeric cells of male recipients were observed in 14 to 20% of myocytes, coronary

arterioles, and capillaries.[43] Chimeric cells were positive for stem cell markers and early heart cell differentiation markers. Recipient chimerism was, however, not related to clinical outcome.

The results outlined above suggest that precursor cells migrate from the recipient into the graft, a process possibly triggered or enhanced by donor tissue damage. However, in many of those cases studied solely by fluorescent in situ hybridization (FISH), it is plausible that the male cells found in female donor hearts may in fact be derived pre-implantation, i.e., via male offspring of female allograft donors.[44]

Pregnancy-Induced Immunization and Fetal-Maternal Microchimerism

The most common natural source of microchimerism is pregnancy. The maternal-fetal interface in the placenta harbors both cells from the mother and from the fetus. Progenitor cell trafficking over the placenta may induce microchimerism for a prolonged period of time after birth in the mother and the child.[45-47] Maternal immune cells may become sensitized to mismatched inherited paternal antigens (IPA) expressed by cells or cellular debris acquired from the developing fetus. This is reflected by the formation of HLA alloantibodies to IPA in a significant proportion of parous women.[48-50] These antibodies are often directed against mismatched HLA-A, -B and –DR antigens, which are not expressed on trophoblast cells, the fetal cell layer which forms the border between mother and child. Therefore it is most likely that fetus-derived chimeric leukocytes are responsible for the induction of these alloantibodies. In addition, the continuous release until birth of placental antigens into the maternal circulation facilitates the systemic induction of maternal effector type as well as regulatory type T cells.

Human fetal or maternal microchimeric cells can be found in circulating blood cells such as granulocytes,[51] lymphocytes,[52] monocytes,[53] and myeloid dendritic cells[54,55] as well as in organs and tissues[56] (**Table 1**). While blood-borne CD45+ chimeric cells are obviously derived from engrafted hematopoietic stem cells,[57,58] the origin and characteristics of the stem cell giving rise to tissue-resident microchimerism, as found in bone marrow, endocrine organs, and other tissues (reviewed in ref. 59) is still unclear. Three candidates have been suggested in this context: mesenchymal stromal cells,[60] pregnancy-associated progenitor cells,[61] and transdifferentiating hematopoietic stem cells.[62]

Using quantitative PCR technology for the detection of nonshared polymorphic HLA genes unique to mother- and fetus-derived cells, it was shown that adult women often carry multiple types of naturally acquired chimeric cells derived from different sources, i.e., from their mother and from their offspring. Addressing the prevalence of these two types of chimeric cells specifically within short-lived and continuously replenished cells of myeloid origin, Nelson and colleagues demonstrated that maternal microchimerism is actually more common in granulocytes obtained from adult females than fetal microchimerism.[51] Neither the prevalence nor concentration

of fetal cells in the maternal circulation seems to be affected by the number of pregnancies. In contrast, the prevalence and level of maternal cells was found to be significantly lower in multiparous women.[46] This unexplained decline in maternal microchimeric cells, ultimately resulting in the dominance of fetal microchimerism in time, could be the result of reciprocal alloimmune reactions between fetus- or mother-derived immune cells (graft-graft interactions), resulting in a survival benefit for one of the two parties as is often seen in the setting of double umbilical cord blood (UCB) transplantation.[63,64] Alternatively, host alloimmune responses directed against circulating fetal chimeric cells may additionally lead to a specific loss of tolerance to non-inherited maternal antigens (NIMA) expressed by the maternal grandmother; the latter situation could eventually lead to specific eradication of HLA class I^{low} hematopoietic stem cells of grandmother origin by host HLA class I-restricted effector cells, while leaving NIMA^{neg} fetal stem cells untouched.

Maternal microchimerism and tolerance to NIMA in the offspring

In 1953, Billingham and colleagues stated that they "are inquiring into the possibility that [actively acquired tolerance] may occur naturally by the accidental incorporation of maternal cells into the fetus during normal development."[6] Fifty-five years later, Mold and colleagues showed that maternal cells engraft in fetal lymph nodes after having crossed the placenta,[65] leading to 0.0035–0.83% of cells in mesenteric lymph nodes being of maternal origin. Specific tolerance of the child's immune system to non-shared NIMA is maintained long after birth.[65,66] Noteworthy, mesenteric lymph nodes belong to a set of organs and tissues collectively termed the mucosa. Natural exposure of naïve T cells to (allo)antigens encountered at the mucosa or in mucosa-draining lymph nodes generally leads to the induction of immunological tolerance. This may explain why successful establishment of NIMA-specific tolerance in newborn mice depends on maternal cells acquired both through placental exchange during fetal development and through nursing after birth.[67,68]

The processes underlying neonatal tolerance induction to NIMA in humans are poorly understood.[69] Mold and colleagues demonstrated that chimeric maternal lymphocytes detectable in fetal mesenteric lymph nodes are protected from immune-mediated destruction by the presence of high numbers of fetal Tregs.[65] Whereas fetal T cells show a proliferative response when combined in vitro with APC from the blood of unrelated adult donors, they do not respond against the NIMA expressed by maternal cells.[65] In the latter case, fetal alloreactive T cell expansion is suppressed by fetal CD4$^+$CD25^{high}Foxp3$^+$ Tregs. In addition to the site where microchimeric cells are encountered, also the type of chimeric cells could be instrumental for the induction of NIMA-specific tolerance. Burlingham and coworkers proposed that maternal MHC class II^{pos} cells from hematopoietic origin such as dendritic cells and macrophages are instrumental for presentation of non-shared maternal antigens to NIMA-specific fetal CD4$^+$ Treg.[68] The potent suppressive function of these NIMA-specific CD4$^+$ Treg was elegantly demonstrated in transplant models wherein NIMA-expressing heart allografts were protected from rejection when implanted in offspring mice, which had been exposed prior to transplantation to the same NIMA antigen as present on the heart allograft during pregnancy and neonatal life.[70,71] Besides dendritic cells, also circulating T cells have been associated with allograft tolerance induction in mice and humans.[54,72,73] Whether single-cell type chimerism or multilineage chimerism, resulting in co-existing myeloid and lymphoid chimeric cells, is required for successful induction and life-long maintenance of alloantigen-specific tolerance[74] remains to be studied in more detail. Based on the identification of CD8$^+$ T regulator cells specific for NIMA presented in the context of maternal MHC class I alleles,[66] it should be noted that also maternal chimeric cells lacking MHC class II expression, i.e., tissue-resident somatic cells, may be involved in the induction of Treg.

In a more recent study Mold and colleagues showed that fetal naïve CD4$^+$ T cells are much more responsive to stimulation with allogeneic cells, but more prone to develop into CD25$^+$Foxp3$^+$ Tregs, compared with adult CD4$^+$ T cells.[75] The findings in humans described above have been confirmed in mice. While fetal tolerance to NIMA seems to be grossly mediated by the effects of alloantigen-specific Tregs,[67] also B cells may become unresponsive to NIMA during fetal life: in 26 highly-sensitized patients awaiting a kidney transplant, a preferential non-responsiveness of alloantibodies to NIMA was found.[76] Thus, several studies have provided evidence that natural exposure to maternal microchimeric cells favors the induction of NIMA-specific tolerance in the offspring.

Priming to NIMA

In contrast to development of NIMA-specific tolerance, there is also evidence that exposure to NIMA can lead to alloantigen-specific priming.[66] It has been proposed that the quality and quantity of NIMA exposure determines whether tolerance or priming will occur.[69] In this setting, tolerance may develop as a result of prolonged exposure to NIMA, both during pregnancy and thereafter during prolonged periods of breastfeeding. In support of this concept, oral exposure of mice to NIMA by breastfeeding led to improved outcome of offspring-to-mother bone marrow transplantation later in life.[77] Furthermore, whereas chronic exposure to low doses of antigen often leads to development of Tregs[78] or to cytotoxic T cell non-responsiveness, small doses induce specific cytotoxic T cell priming.[79,80]

Detection of functionally different types of T cells directed against fetal IPA in parous females

As extensively studied in murine pregnancy models, maternal Treg imprinting is essential for normal pregnancy outcome after both syngeneic and allogeneic pregnancy.[81] In the human setting, fetal-specific CD8$^+$ T lymphocytes have been observed in half of all pregnancies analyzed.[82] These T cells typically become detectable after the first trimester, display an effector memory phenotype, and persist in the postnatal period. To study the functional features of such pregnancy-induced T cells, several studies analyzed the prevalence and functional properties of T cells specific for Y chromosome-encoded minor histocompatibility antigens. These so called HY antigens are small peptides derived from intracellular proteins, which are presented by HLA class I or class II alleles at the surface of male cells. To date, many different

HY peptides have been identified which cause potent alloimmune responses in gender mismatched transplantation settings. Using a HY peptide/multimer-based sorting procedure, HY-specific T cells with classical cytolytic function have been isolated from blood samples collected either during[82] or after[66,83,84] male pregnancies. Using the trans-vivo delayed-type hypersensitivity (tvDTH) assay,[85] also the presence of HY-specific Tregs in women with a documented pregnancy history has been reported.[66] This assay detects the presence of Tregs, which are able to suppress footpad swelling induced by chemokines and cytokines released by co-activated recall antigen-specific memory T cells. In approximately half of the parous women analyzed, HY-specific Tregs could be detected up to several decades after the delivery of a son. Some women show profound HY-specific regulation, but others show only marginal suppression of recall-antigen-induced foot pad swelling responses in the presence of HY peptide(s).[86] It is therefore tempting to speculate that either HY-specific regulatory T cells or cytolytic T cells dominate in some women with sons, but that these functionally different types of T cells co-exist in others.

The alloimmune status of individuals, who bear microchimeric cells

As discussed above, fetal and maternal microchimeric cells are found regularly among blood-borne cells and tissue-bound cells. By studying the offspring from specifically selected mouse breeding models, it has become clear that the levels of maternal microchimerism in blood and various organs such as the heart is directly correlated with the presence of CD4^{+} Tregs directed against NIMA antigens that have been encountered during fetal or neonatal life.[67] Also in humans the putatively lifelong presence of maternal microchimeric cells is extensively documented. Likewise, the presence of male microchimerism in adult women has been widely reported. Despite the relatively high frequencies of chimerism detected in many women, there does not seem to be a correlation between the level or subtype(s) of circulating IPA-expressing chimeric cells and the presence of IPA-specific Treg.[86] Interestingly, male chimerism is not necessarily restricted to women with male offspring, given that trans-maternal flow of male cells derived from a former pregnancy or from a twin sibling who died in utero can be transferred during a next pregnancy.[47,55,87] Consequently, female cord blood units may comprise functional T cells directed against alloantigens, which are not expressed by the mother but which have been encountered through trans-maternal cell flow of chimeric cells.[55] Likewise, the alloimmune T cell repertoire of multiparous women may contain considerable precursor frequencies of cells directed against the various mismatched HLA alleles expressed by either the maternal grandmother or by her offspring, or against mismatched minor histocompatibility antigens presented by shared HLA antigens.

Potential Clinical Benefits of Pre-Transplant Existing Microchimerism

Pre-transplant induced alloimmunization to mismatched alloantigens expressed by microchimeric cells may affect transplant outcome when such individuals enroll in a transplantation program. In case of durable maternal-specific tolerance in the child, this may have clinical consequences in case the child undergoes a transplant procedure later in life. Indeed, this has been found to be the case in the kidney transplant setting: graft survival was significantly higher in recipients of kidneys from siblings expressing NIMA than in recipients of kidneys from siblings expressing non-inherited paternal antigens (NIPA).[88] The tolerogenic effect of having encountered NIMA prior to transplantion may be employed in the setting of unrelated donor and cord blood HSCT,[89–91] for instance by including the NIMA in matching criteria.

HSCT using adult donors

Allogeneic HSCT is the curative treatment option for many hematological malignancies as well as for several life-threatening hematological diseases. The success of this treatment is largely determined by selection of the optimal stem cell donor, according to HLA typing of both the stem cell recipient and donor. One of the major causes of morbidity and mortality following adult or pediatric HSCT is GvHD, which may even occur in the case of full HLA compatibility between donor and recipient. Mismatched minor histocompatibility antigens expressed by the transplant recipient, but not the donor, are evoking the sometimes devastating immune responses in sibling transplantation. The involvement of alloreactive T cells in this process is illustrated by the detection of minor histocompatibility antigen-specific T lymphocytes in blood[92] and tissues[93] obtained from patients who developed GvHD after infusion of a Tcell-containing graft. GvHD can be even more severe in case of HLA mismatching, i.e., after transplantation with HLA-haploidentical donors (donors that share one haplotype with the recipient, e.g., parents to children or vice versa or transplants between haploidentical siblings). The so called graft-vs.-leukemia (GvL) reactions represent the other aspect of immunologic reactions mediated by the donor-host interactions. GvL reactions may contribute to regression of leukemia or prevention of a relapse after allogeneic transplantation. Lastly, rejection of a donor graft can be mediated by immunologic reactions against mismatched minor or major histocompatibility antigens.

Donor parity is a risk factor for the development of GvHD in both male and female recipients of a transplant from a HLA-identical donor.[94] Interestingly, donor or recipient parity is also frequently associated with GvHD seen after HSCT between monozygotic twins, a setting in which neither HLA nor minor histocompatibility antigen disparities are involved.[95] The donor parity effect has been attributed to the presence of fetal microchimeric cells in female donors. Such "foreign" microchimeric cells likely act as triggers of alloimmune responses by donor T cells, but the exact immunological mechanism by which fetal microchimerism could increase the GvHD risk in the recipient has yet to be determined.

Superior transplant outcome in recipients of maternal grafts

The persistence of bidirectional microchimerism between mother and child may have implications in family HSCT transplantation settings. Three studies have shown that in haploidentical transplantation, the parental origin of the
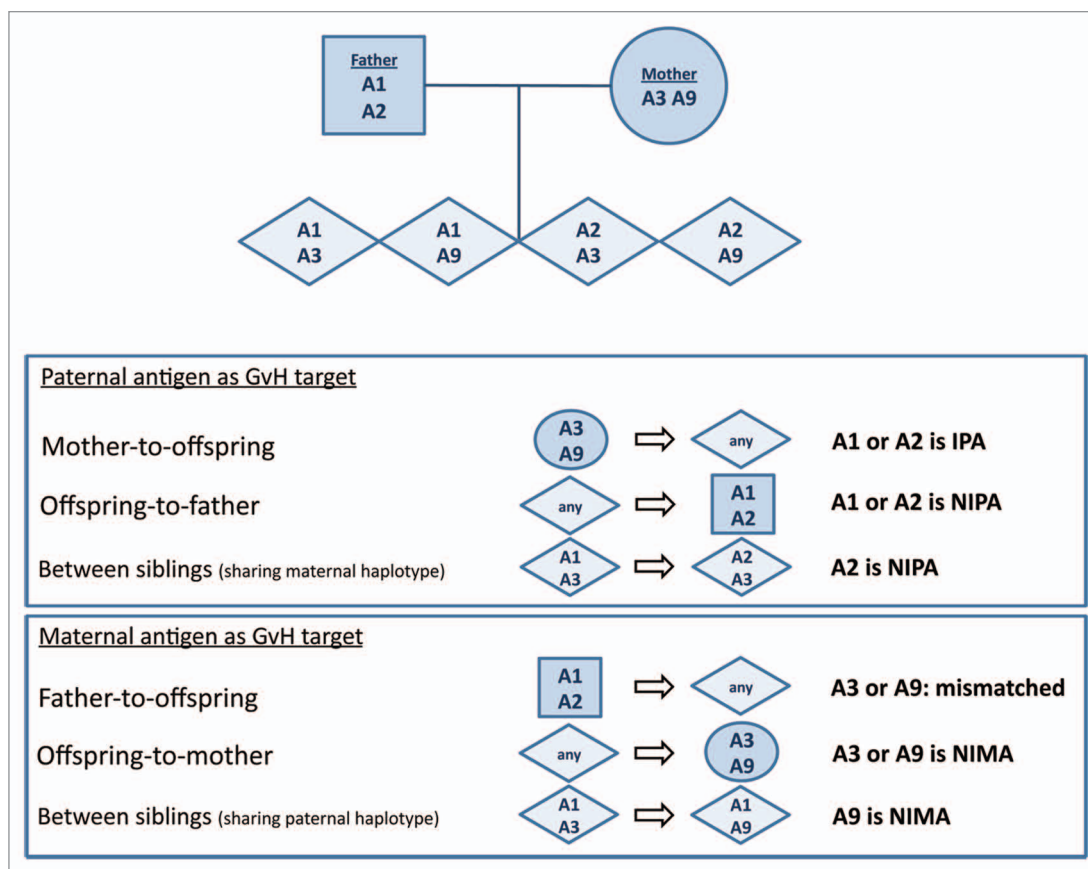
Figure 1. Exemplary scheme of shared and non-shared HLA antigens in the mother, the father, and the offspring. In this example the father has HLA-A1 and -A2 and the mother has HLA-A3 and -A9. In hematopoietic stem cell transplantation, the inherited paternal antigen (IPA), non-inherited paternal antigen (NIPA), and non-inherited maternal antigen (NIMA) have been indicated in case of a mother-to-offspring, offspring-to-mother, father-to-offspring, offspring-to-father or offspring-to-offspring combination.

mismatched HLA haplotype determines outcome. In the first study, Van Rood and colleagues analyzed a subset of haploidentical transplants (father-to-offspring, mother-to-offspring, or among HLA haploidentical siblings; see **Fig. 1** for exemplary scheme).[96] When the transplantation was performed among siblings who shared a paternal haplotype, the incidence of acute GvHD was significantly lower compared with all other donor-recipient combinations. This was attributed to the fact that the target for GvHD reactions was the maternal NIMA, to which the donor had been rendered tolerant during fetal life. All other situations (father-to-offspring, mother-to-offspring or transplants between siblings mismatched for the maternal NIPA) were associated with a higher incidence of acute and chronic GvHD. Notably, in the latter cases there had been no intrauterine exposure of the donor cells to GvHD targets present in the recipient. The incidence of graft rejection was lower when using maternal grafts compared with paternal grafts.

In a Japanese study of T-cell-replete transplants, donors were selected to have detectable microchimerism (by PCR) for antigens present in the recipient.[97] The donors were either siblings that shared paternal antigens (GvHD target was NIMA), mothers donating to offspring, or children who were used as donors for their mother. Fathers were not used as donors, nor were siblings

who shared a maternal haplotype. Among 35 donor/recipient pairs, 15 were transplants from mother-to-offspring and the GvH reactivity was against the paternal HLA of the recipient (the IPA). In 20 cases, a transplant from offspring was infused in mother or in NIMA-incompatible offspring. The latter 20 cases had a significantly reduced risk of grade III-IV acute GvHD, presumably because exposure in utero to the NIMA had led to the development of NIMA-specific immune regulatory cells in the donor. Vice versa, it was proposed that the maternal grafts had developed persisting life-long immunity against IPA due to previous exposure of the maternal immune system to fetal antigens during pregnancy. Consequently, the presence of pregnancy-induced alloimmune effector T cells in the grafts of maternal donors may have caused more GvHD.

The third study by an Italian group emphasized the same principle in the setting of T-cell-depleted HSCT, which significantly reduces the GvHD risk. Pharmacological post-transplant immune suppression is not used in this transplant procedure. Comparing transplant outcomes from maternal and paternal grafts, they found that maternal grafts were associated with reduced rates of disease recurrence.[98] This was seen in both female and male recipients. The improved overall survival mainly resulted from a lower incidence of relapse of

malignant hematologic disease, as the incidence of acute GvHD was not significantly different between maternal and paternal grafts. It was proposed[98] that in this case the primed maternal grafts mainly induce a beneficial GvL effect. GvHD is avoided because of profound T cell depletion. The authors speculate that the few T cells transferred with the graft (in this study, a median of 10^4/kg, i.e., a total of 0.5–1 million cells per graft), may have undergone unopposed proliferation after infusion in the recipient by virtue of the absence of pharmacologic GvHD prophylaxis. Two other retrospective studies confirm the superior survival of blood or marrow stem cell recipients given maternal grafts over recipients given paternal grafts.[97,99] Whether pregnancy-induced memory T cells specific for shared paternal major or minor histocompatibility antigens[83,100] expressed by the transplant recipient's leukemic cells are among the expanding female donor-derived T cells remains to be studied.

Umbilical cord blood transplants

UCB transplantation is increasingly used in transplantation of children and adults, who lack a matched unrelated donor or extended family donor. Compared with transplants from adult donors with similar degrees of HLA disparity, UCB transplantation is associated with a reduced rate of disease recurrence and a decreased incidence of GvHD.[101] The relapse rate may even be lower after double UCB transplantation than after a single UCB transplantation, although the latter hypothesis was not confirmed in a recent randomized study.[102] The reason for the reduced rate of disease recurrence after UCB transplant has remained elusive, but may be indirectly related to maternal microchimerism. Emerging evidence indicates that the fetal immune system, and thus relevant for UCB grafts, is geared toward peripheral tolerance maintained by Tregs. This situation is thought to result in lifelong tolerance to NIMA encountered during fetal/neonatal life. Fetal tolerance to NIMA may be an important mechanism by which the GvHD risk is reduced after UCB transplantation. Van Rood and colleagues analyzed a group of UCB transplant recipients, of whom the complete HLA typing of the infant (donor of the UCB unit) and of the infant's mother were available.[103] Reexposure of cord blood to NIMA expressed by the recipient of the UCB graft improves transplant outcome in hematological malignancies. Most of the UCB recipients were HLA-mismatched with their donors at one or several antigens. But in some cases the HLA mismatch was identical to an HLA antigen present in the mother of the infant donor (NIMA match). In other cases, the mismatched HLA antigen was not present in the donor's mother (no-NIMA match). Treatment-related mortality was significantly lower in the recipients of a NIMA-matched transplant, in part because of faster engraftment, but also because of a lower relapse rate. The authors speculated that cord blood, thought to be generally tolerant toward NIMA, might sometimes be sensitized, as was described for minor histocompatibility-antigen HA-1^H specific T cells.[104] In a more recent case-control study, the outcome of 48 NIMA-matched UCB transplants was compared with that of 116 non-NIMA-matched UCB transplants.[90] The groups were matched for important covariates. Transplantation-related

mortality was lower and overall survival significantly higher after NIMA-matched UCB transplantation compared with NIMA-mismatched UCB transplants. The effect of NIMA matching on relapse rate was not analyzed in this study.

Antileukemia effect and anti-IPA immunity exerted by microchimeric cells present in cord blood grafts

Van Rood and colleagues retrospectively analyzed in a large number of UCB transplants the HLA type of both mother and UCB, and deduced the IPA of the UCB.[105] Transplants with IPA-targeting UCB were identified, in which the maternal lymphocytes would be primed against the IPA of the UCB and also against cells of the recipient who expresses the same IPA. Such was the case in most donor-recipient pairs. They compared the outcome of this group with the outcome of a limited number of cases where there was no IPA reactivity. They found an increased rate of disease recurrence in the latter group. Importantly, the decreased leukemia recurrence rate after IPA-targeted UCB transplantation was not associated with increased GvHD, possibly resulting from the effectiveness of co-transferred NIMA-specific fetal Tregs. Similar to possible mechanisms explaining the beneficial outcome of patients with a maternal graft, it was speculated in this particular paper[105] and in related editorials,[106,107] that maternal cells—earlier being primed against the IPA of the child and present in the UCB graft—confer a potent GvL effect. The results from these consecutive studies provide the first epidemiological evidence that anti-IPA immunity by maternal immune cells may play a role in the control of relapse in acute myeloid leukemia and acute lymphatic leukemia after unrelated cord blood transplantation.

The major challenge to date is how to address the actual role of microchimeric cells in UCB, a question that can only be addressed after isolation of these cells from the host background. One of the first questions arising from the proposed concept of antileukemia effects by maternal cells presumably present in cord blood grafts is which type of maternal cells is doing the job. These could be both CD4$^+$ and CD8$^+$ T cells, the importance of which has been described for GvL reactivity in mice.[108,109] Furthermore, NK cells may contribute to the anti-leukemia effect (reviewed in ref. 110). Since anti-leukemic effects of NK cells are probably less prominent than those of T cells, the GvL effect by NK cells may come to the foreground after haploidentical transplantation in combination with rigorous T cell depletion. The same may hold true for UCB transplantation, given the significant delay in T cell recovery in this setting.[111] Second, do microchimeric maternal cells require HLA specificity for targeting leukemic cells in the recipient after transplantation? Van Rood and colleagues showed that HLA antigens, i.e., those that the recipient shared with the cord blood, represent one target of relapse-reducing immunity. It has been proposed that eradication of leukemic cells results from Fas- or perforin-mediated mechanisms that are typically displayed by lymphocytes.[110,112] Such mechanisms may even be more prevalent in situations of HLA-related, maternal-derived immunity. It is also possible that the GvL effect is established due to the mere presence of sensitized maternal cells within the graft, which are more immunogenic than the cord blood cells.

Table 2. Techniques for detecting of microchimerism[a]

Technique	Sensitivity[b]	Advantages	Disadvantages
FISH	10^{-2}–10^{-6} (depending on target and method)	Identification in context of tissue morphology	Takes lot of time for analysis (up to 2 wk per sample) in case of high sensitivity
Nested PCR	10^{-4} or lower	Higher sensitivity than direct PCR	High chance of false positivity by contamination; not quantitative
Real-time qPCR	5×10^{-4}–10^{-5} (depending on target)	Quantitative	Detection at low percentages requires running of multiplicates
Clamp PCR	10^{-4}, down to 10^{-5} or lower	Reduction of background levels	Cumbersome to find optimal probes; LNA modifications costly; novel methodology
WGA+qPCR	similar as qPCR	Allows more analyses per sample	No enhancement of sensitivity; requires additional manipulation of the sample
Single cell analysis	-	Information per cell	Cells need pre-enrichment; limited # of cells in analysis; may be most suitable for RNA
Digital PCR	5×10^{-5}–5×10^{-6}	Reliable and reproducible; background copies reduced	Conventional qPCR assays need to be optimized for digital PCR
HLA FACS sorting	~10^{-4}	Generates viable chimeric cells for functional studies	Limited sensitivity; requires access to high-quality, allele-specific HLA mAbs

[a]FISH, fluorescence in situ hybridization; WGA, whole genome amplification; PCR, polymerase chain reaction; qPCR, quantitative PCR; LNA, locked nucleic acid; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody. [b]10^{-4} = 1 in 10 000 cells (0.01%); 10^{-5} = 1 in 100 000 cells (0.001%); 10^{-6} = 1 in 1 000 000 cells (0.0001%).

Detection and Enrichment of Microchimeric Targets

In the animal and human solid organ transplantation setting, chimerism could often not be detected by the serologic assays that were available at the time. Only after the introduction of the polymerase chain reaction (PCR), which led to a greatly enhanced sensitivity of chimerism measurement, it became possible to detect rare donor cells in the circulation and organs of the transplant recipient. An essential step in investigating the clinical consequences of microchimerism is the availability of sensitive tools to detect microchimerism. Furthermore, isolation of viable microchimeric cells from the host is essential for further characterization and functional analysis of such cells. In the next paragraphs an overview of the different methodologies used is given. We have summarized these methodologies in **Table 2**, along with the sensitivity, advantages, and disadvantages.

In situ detection of tissue-resident microchimeric cells

Fluorescence in situ hybridization (FISH) is a popular technique to identify microchimeric targets: chimeric cells can be identified within the context of tissue morphology and localization. Traditionally, sex chromosome-based XY-FISH has been used for chimerism analysis, whereby male cells are identified in a female genetic background. FISH can give false-positive results in rare cell conditions. The use of two different Y probes[113] and the application of rehybridization with X and Y chromosomes with reverse colors[114] may enhance reliability. Within the context of autoimmune disease, the group of Deforce has applied XY-FISH to count the number of Y-positive, fetal cells in women.[115] With this approach they were able to distinguish 8–29 fetal cells/10^6 maternal cells in women with autoimmune disease from 0–2 fetal/10^6 maternal cells in healthy, pregnant women, either before delivery or several months postpartum.

PCR-based methodologies

Both classical and real-time quantitative PCR (qPCR) techniques have been used for detection of (micro)chimerism.[116-119] Increased sensitivity may be achieved when the preliminary PCR is followed by second-round PCR with sequence-specific primers that target the first amplicon.[120-123] However, as we (M.M., B.M.K.-M., J.J.M.D., M.E.; unpublished observations) and others[124,125] have found, nested PCR is prone to decreased specificity and to contamination by nonspecific PCR products, which results in false-positive results.

Real-time qPCR offers an accurate and rapid tool compared with conventional PCR analysis of DNA tandem repeats, which is classically used to assess macrochimerism levels after HSCT.[126] Multicopy genes localized on the Y-chromosome represent suitable targets for reproducible and sensitive analyses by qPCR.[29,57,117,127,128] Its application is, however, restricted to sex-mismatched combinations, whereby male DNA targets are targeted in a female host. DYZ1 primers target a Y-chromosome selective sequence that is found 8 times in total in the DAZ2, DAZ3, and DAZ4 genes. The benefit of this assay is that it offers a relatively high sensitivity of 0.001% (1 in 100 000 cells detected; 10^{-5}). This sensitivity is approximately 10-fold higher than that of single-copy genomic targets such as mismatched HLA genes and insertion/deletion regions.

One cell contains an estimated 7 pg DNA. A typical input of 0.5 μg DNA per conventional qPCR assay is expected to contain approximately 70 000 amplifiable targets. With 1 in 100 000 microchimeric targets, this would mean that on average 0.7 copies of DNA are available in the sample for amplification. According to expected stochastic results predicted by the Poisson equation, one copy is present in 82% of the time.[138] For conventional qPCR, this means that in practice a single sample
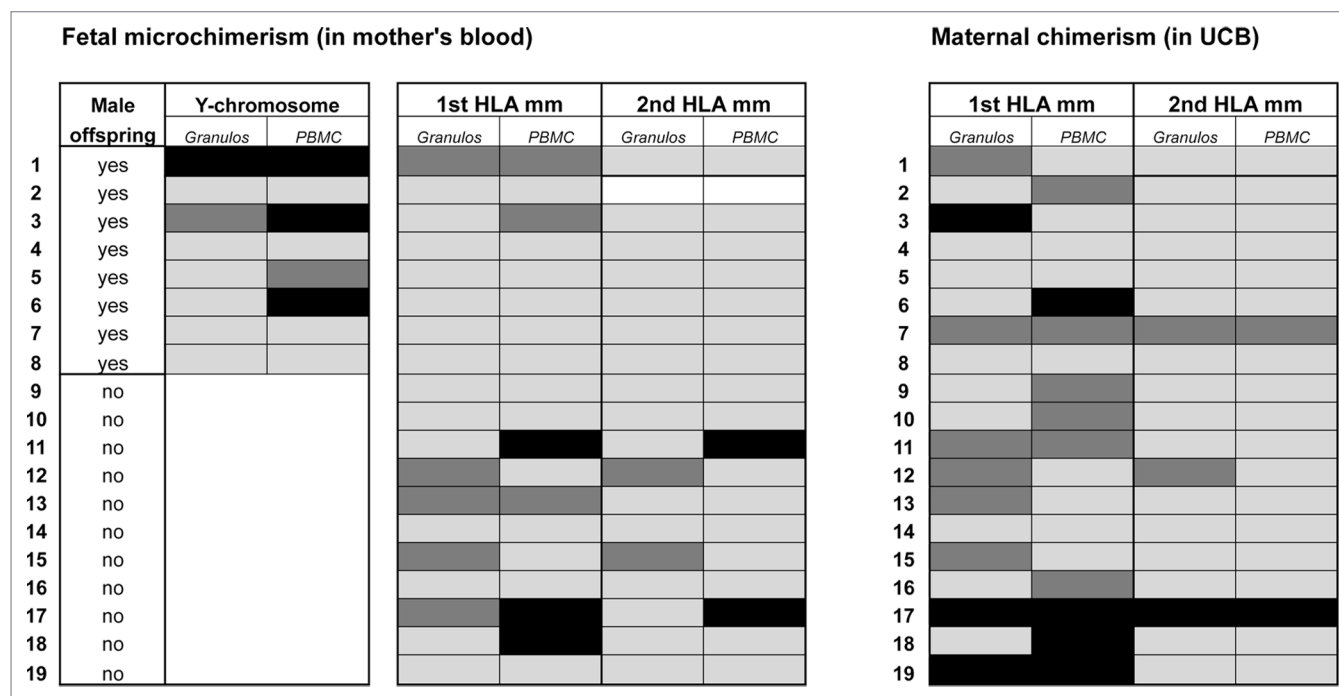
Fetal microchimerism (in mother's blood)

	Male offspring	Y-chromosome Granulos	Y-chromosome PBMC	1st HLA mm Granulos	1st HLA mm PBMC	2nd HLA mm Granulos	2nd HLA mm PBMC
1	yes	positive	positive	weak	weak	neg	neg
2	yes	weak	positive	neg	neg	—	—
3	yes	weak	positive	neg	weak	neg	neg
4	yes	neg	neg	neg	neg	neg	neg
5	yes	neg	weak	neg	neg	neg	neg
6	yes	neg	positive	neg	neg	neg	neg
7	yes	neg	neg	neg	neg	neg	neg
8	yes	neg	neg	neg	neg	neg	neg
9	no	—	—	neg	neg	neg	neg
10	no	—	—	neg	neg	neg	neg
11	no	—	—	neg	positive	neg	positive
12	no	—	—	weak	weak	neg	neg
13	no	—	—	neg	neg	neg	neg
14	no	—	—	neg	neg	neg	neg
15	no	—	—	weak	neg	weak	neg
16	no	—	—	neg	neg	neg	neg
17	no	—	—	neg	positive	neg	positive
18	no	—	—	neg	positive	neg	neg
19	no	—	—	neg	neg	neg	neg

Maternal chimerism (in UCB)

	1st HLA mm Granulos	1st HLA mm PBMC	2nd HLA mm Granulos	2nd HLA mm PBMC
1	weak	neg	neg	neg
2	positive	weak	neg	neg
3	positive	neg	neg	neg
4	neg	neg	neg	neg
5	neg	neg	neg	neg
6	neg	positive	neg	neg
7	weak	weak	neg	neg
8	neg	neg	neg	neg
9	neg	weak	neg	neg
10	neg	neg	neg	neg
11	weak	positive	weak	neg
12	weak	neg	weak	neg
13	weak	neg	neg	neg
14	weak	neg	neg	neg
15	weak	neg	neg	neg
16	weak	neg	neg	positive
17	positive	positive	positive	positive
18	positive	neg	neg	neg
19	positive	positive	neg	neg

Figure 2. Fetal and maternal microchimerism in mother-child combinations. Presence of microchimerism was investigated using Y-chromosome-specific and HLA-directed primer sets on DNA from umbilical cord blood (UCB) and peripheral blood of 19 mother-child combinations. Mean gravidity of the women was 2.8 ± 1.5, and mean parity was 1.4 ± 0.8. In most cases, blood was collected at a time point between 1 d before delivery and 1 d after delivery. Blood cells present in the Ficoll interface (indicated as "PBMC") and in the Ficoll pellet (indicated as "granulocytes") were separately processed for DNA extraction and microchimerism analysis by qPCR. Black boxes: positive signal (microchimerism detected). Dark gray boxes: weakly positive signal. Light gray boxes: negative signal (no microchimerism detected). Empty boxes: no informative marker available.

should be analyzed in multiplicate to detect microchimerism with a frequency ≤ 0.001%.

HLA represents a broad applicable target for microchimerism detection,[116,117,129,130] as they are highly polymorphic[131] and thus frequently mismatched between two individuals, irrespective whether there is a gender mismatch or not. Over the past years, several research groups, including those from Lee Nelson and Nathalie Lambert (Seattle WA) and from Diana Bianchi (Boston MA), have been developing HLA-allele specific qPCR assays. Using both DYZ1 and in-house developed HLA-directed primer sets, we have investigated presence of microchimerism in blood lymphocytes of 19 mother-child combinations in the PBMC fraction (**Fig. 2**). With only DYZ1, male microchimerism was detected in 4 out of 8 (50%) mothers who gave birth to a son. In the other 11 cases, DYZ1 was regarded as being not informative, since the women gave birth to a daughter. In line with other reports,[132,133] we observed in a more recent analysis of a larger group of women with no sons, i.e., nulliparous women or females who had given birth to a daughter, a fairly high percentage of women displaying male microchimerism.[86] With HLA-directed primers, 6 out of 19 (32%) showed weak or clear positivity of fetal microchimerism (**Fig. 2**). Two of these (#11 and #17; 11%) could be verified by a qPCR assay that targets a second fetus-specific HLA allele. Maternal microchimerism in the PBMC fractions of the 19 corresponding UCB samples was seen more frequently, namely in 10 cases (53%). Two of these (UCB7 and UCB 17) could be verified using a second HLA target.

Insertion-deletion (indel) polymorphisms[118,119,134,135] localized throughout the genome may represent complimentary targets for PCR, which in contrast to the HLA complex may enable clearer distinction of the specific allele from the off-target al.ele. Other targets that can be used to discriminate the chimeric population from a host are short-tandem repeats, genes encoding the Rhesus system, minor histocompatibility genes, and the killer-cell immunoglobulin-like receptor (KIR) genes. Mitochondrial DNA (mtDNA) may be a useful target for detecting microchimerism. This type of DNA is contained in the mitochondria on an extra-chromosomal and circular genome, and holds highly polymorphic regions in the displacement loop.[136] Since every cell contains hundreds of mitochondria, each having many circular chromosomes, mtDNA represents a naturally enriched source of target DNA for microchimerism detection in the transplantation setting. Mitochondrial DNA is inherited solely from the mother, and thus its measurement cannot be applied for addressing pregnancy-related maternal and fetal microchimerism.

Enhanced PCR assays by probe modification

Novel PCR-based technology and modification of primer/probe sequences may further enhance sensitivity and specificity of PCR assays aimed at detecting microchimerism. Much of this technology has been pioneered in the field of cancer biology for detecting low-level cancer-related mutations.

Inclusion of one or more locked nucleic acid (LNA) modifications within primer sequences has resulted in improved results. LNAs enable high affinity binding of the probe to the complementary sequence, leading to enhanced sensitivity

and specificity of the PCR assay. A relatively novel strategy is detection of the specific (microchimeric) allele in combination with blocking of amplification of the off-target (host) allele. CastPCR (Life Technologies) is aimed at detection of somatic mutations in cancer genes. Allele-specific qPCR primers that detect the mutant allele are combined with allele-specific Minor Groove Binder (MGB) blocker oligonucleotides, which suppress non-specific amplification of the off-target (wild type) allele. It is claimed that the assays can detect as few as 1 to 5 mutant copies in up to one million of background copies. PCR clamping with peptide nucleic acid (PNA)- or LNA-modified oligomers allows sensitive detection of targets present at a low frequency, and at the same time inhibits amplification of non-target (background) DNA.[137] The combination of allele-specific priming, competitive probe blocking, and melt curve analysis has allowed single copy detection of variant alleles down to a sensitivity of at least 0.001% (10^{-5}).[138] The methodological approaches outlined above may be customized to detect appropriate target al.eles for application in the microchimerism field.

Nucleic acid pre-amplification and single cell analysis

To multiply the amount of starting material before allele-specific qPCR analyses, whole-genome amplification with multiple displacement amplification (MDA) technology can be performed. Application of this technique gives up to 10 000-fold amplification of the whole genome.[139,140] Due to the unbiased amplification of the genomic DNA, more tests can be performed by qPCR on one sample compared with situations without such pre-amplication. This may be a benefit, especially in the light of the need for running multiplicates of a certain microchimeric target al.ele in the same sample.

Since a cell contains multiple copies of mRNA transcripts, produced from any gene sequence encoded within the DNA, RNA lysates represent a naturally enriched source for the detection of microchimerism. On top of that, starting quantities down to the nanogram or even picogram range of RNA can be amplified in an unbiased manner. Availability of RNA amplication methodology has opened the possibility of expression profiling in single cells. Fluidigm has introduced the C_1 Single-Cell AutoPrep System. Cells need to be enriched first, for example by FACS sorting, and 750–1000 cells (in 5 μL) are then entered into the microfluidic system. The C_1 technology allows capturing of single cell in each chamber, the possibility of visual verification under the microscope, and profiling of each of the individual cells. The capillary system, 10 to 25 μm in size, captures single cells into 96 separate chambers, where cDNA is transcribed and pre-amplified. Subsequently, gene expression profiling and even whole transcriptome sequencing can be performed on each individual eluate. AmpliGrid technology (Advalytix) makes use of a similar approach: single cells are deposited into 48 different reaction sites by FACS sorting, which then undergo reverse transcription and real-time qPCR.

Digital PCR: a possible novel tool for microchimerism detection

Digital PCR represents the latest generation in PCR technology: each sample is diluted into partitions (separate reaction chambers), and the number of partitions in which a reaction occurs are counted up.[141] The benefit of ddPCR over conventional qPCR is enhanced accuracy and reproducibility. Fluidigm and Life Technologies provide a system of reaction chambers on chips (microscope slides) or plates. In droplet digital (dd)PCR (Bio-Rad and RainDance) the nucleic acids in the lysate are dispersed into thousands to millions droplets at a concentration of less than one genome equivalent per droplet. The droplets are subsequently transferred to tubes, and subjected to PCR to analyze per droplet whether a reaction has occurred (**Fig. 3A**). A Poisson algorithm is then applied for determination of absolute copy numbers.

Digital PCR has been used to analyze rare DNA targets in samples, e.g., for detecting fetal DNA in maternal plasma[142] and viral marker genes in single bacterial cells.[143] This "needle-in-a-haystack" strategy makes the system the perfect tool to detect low numbers of microchimeric targets. Detection of chimeric DNA targets with a frequency of 1 in 10 000 background copies (0.01%; 10^{-4}) with 95% reliability requires screening of at least 30 000 copies (~200 ng DNA) in total. When aiming to detect lower microchimeric frequencies, the amount of DNA as input needs to be increased to 0.5–1 μg and distributed over two to four wells. The number of positive and negative partitions from different wells can be added up and merged. Researchers have even been able to detect one mutant allele in a background of 200 000 wild type alleles (0.0005%; 5×10^{-6}).[141,144]

Using ddPCR for the detection of Y chromosomes, we monthly monitored fetal microchimerism in peripheral blood samples obtained from a pregnant colleague known to carry a male fetus. Y chromosome-specific DNA was first detected in whole blood after 6 mo of pregnancy, and the extent of male DNA further increased over time (**Fig. 3A**). We obtained a sensitivity around 2×10^{-5} for PCRs targeting DYZ1 (**Fig. 3B**, black symbols) or an indel region (S09b; **Fig. 3B**, gray symbols). The multicopy benefit of the Y chromosome targeted by conventional qPCR does not apply for ddPCR. The extent of false positivity in negative controls is reduced in ddPCR compared with conventional qPCR (see **Fig. 3C**), since amplification of genomic targets in separately dispersed partitions increases PCR specificity by isolating the target signal from competing background.

Enrichment of microchimeric cells from bulk populations

With percentages of microchimerism of 0.01 or lower it would not be practically and economically feasible to analyze ≥10 000 single cells in search of that one microchimeric target. Instead, enrichment of chimeric targets may be reached by laser pressure catapulting of individual cells in a cytospin. Researchers from Austria have used laser microdissection to catapult rare, single candidate target cells from a total cytospin onto AmpliGrid slides.[145-147] To verify the genomic identity of the target cell, it was subsequently subjected to whole genome amplification[146] and DNA fingerprint analysis. DNA profiling on single cells could be used in 86% of the times to verify the genomic background of the cell.[145] Full DNA profiles could be obtained with fewer than 10 cells (~70 pg) as input after on-chip DNA amplification.[148,149]

Once processed for qPCR or in situ analysis, functional studies of chimeric cells are precluded. One method for pre-enriching chimeric cell populations may be to separate cell subpopulations
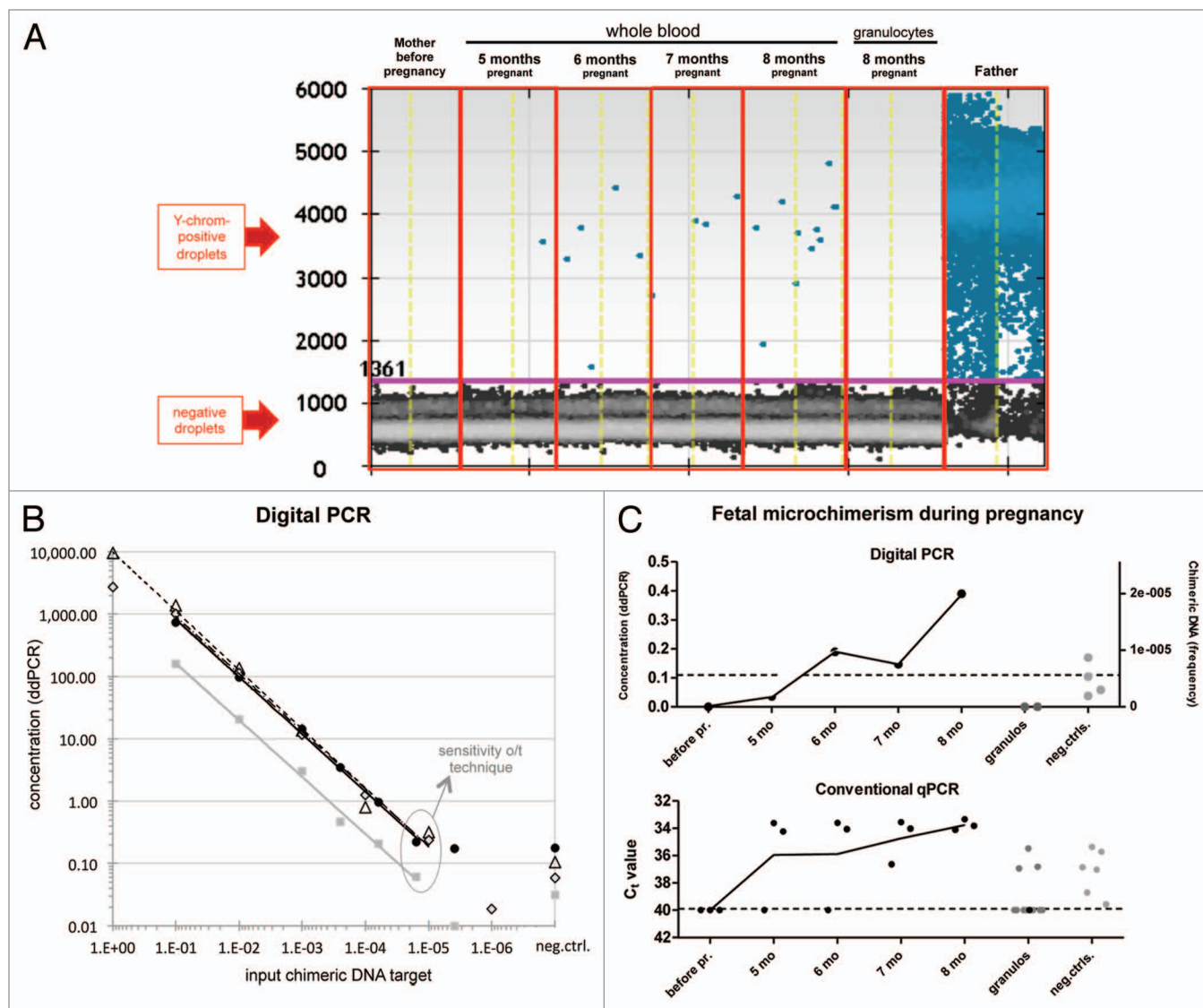
Figure 3. Droplet digital PCR (Bio-Rad, QX100) results for microchimeric targets. (**A**) A pregnant woman was monthly monitored for fetal microchimerism by PCR for the Y chromosome in whole blood and in the granulocyte fraction. Each dot in the picture represents one droplet. The software distinguishes between positive droplets (the upper ones) and negative droplets (the lower ones) for the DNA target of interest. Each condition was analyzed in duplicate, and results were merged in the software. The total input of DNA per reaction was 270 ng. (**B**) DNA from one donor was diluted in DNA from another donor, in different ratios (10^{-1} to 5×10^{-6}). Linear correlation between input and observed percentage was obtained at chimeric frequencies down to 2×10^{-5} (1 in 50 000 cells) for both Y-chromosome- (black circles, triangles, and diamonds) and indel-region (S09b)-targeting PCR assays (gray squares). The standard for the Y target was performed three times, each consisting of two to five replicates. (**C**) Comparison between ddPCR- and conventional qPCR results for the Y-chromosome PCR in pregnancy samples and in negative controls.

from the total lysate using magnetic cell sorting (MACS). This strategy especially holds true when clinical importance of the chimerism is expected to be related to a particular cell lineage where it resides. In such case, lineage-specific analysis of chimerism may be more informative.[150,151] In most samples, purity of >90% was reached after MACS for the T cell (CD3+), monocyte (CD14+), and granulocyte (CD15+) subsets.[152] In the majority of samples the subpopulations provided at least 0.5 ng/μL of DNA, which was a prerequisite for reliable qPCR-based chimerism analysis.[152]

Viable microchimeric cells can also be targeted and separated from the host environment by FACS sorting. Flow cytometry

with anti-HLA mAbs is already used as a monitoring tool for chimerism after HLA-mismatched HSCT.[153] In the late seventies, Herzenberg and colleagues described a FACS enrichment procedure of chimeric cells: anti-HLA-A2 antibodies were used to target fetal cells in a background of maternal peripheral blood lymphocytes.[154] We have developed a set of 120 human HLA-specific monoclonal antibodies in our laboratory. Single HLA mAb labeling of cell populations that are present in a frequency of 0.4% or lower leads to suboptimal separation from the host cells. Double HLA antibody labeling was found to be essential for optimal separation of low-frequency microchimeric cells.[155,156] Purity of the sorted samples was verified by qPCR analysis for

HLA class I and class II alleles.[156] We further applied HLA-targeted FACS sorting on UCB samples to separate microchimeric maternal cells. The methodology is applicable in case the HLA type of the minor cell population is known, for example for microchimerism enrichment in pregnancy and transplantation, and in case the frequencies of microchimeric cells are relatively high, as is often seen in UCB samples. For detection of fetal HLA targets in maternal blood before delivery, one could target HLA antigens from both paternal haplotypes, provided that the antibodies used for labeling the chimeric cells do not cross react as is often the case with HLA-specific antibodies.

Summary and Objectives for Future Research

We aimed to compose a comprehensive overview of the literature on naturally-acquired and transplant-associated microchimerism, and describe its effect on clinical transplant outcome. The results from literature reports on these complex topics are often contradictory. Furthermore, there is a requirement for systematic and in depth studies on the (patho)physiologic mechanisms of microchimerism and corresponding cell types in order to understand the interesting but as yet poorly understood epidemiological data obtained from clinical transplant settings.

Adult and cord blood HSCT may benefit from HLA matching criteria whereby the IPA and NIMA are taken into account. The NIMA effect in solid organ transplantation observed in studies that appeared in the previous century seems to have become less prominent nowadays, probably as a result of the change in the immunosuppressive medication regime over the years. To establish whether persistence of the NIMA effect later in life is enforced by prolongation of microchimeric conditions in the host would be subject of investigation. It remains elusive whether increased frequencies of maternal chimerism in cord blood samples are related to NIMA-specific Tregs. Likewise, several questions remain to be solved around the concept of antileukemia effects by maternal cells with anti-IPA immunity present in cord blood grafts. Do microchimeric maternal cells require HLA specificity for targeting leukemic cells in the recipient after transplantation? And why do the microchimeric anti-IPA-directed maternal cells cause no harm to the cord blood cells present in the same storage bag? Are maternal effector cells actively suppressed by (maternal) regulatory cells, and if so, which circumstances in the recipient could stimulate the anti-leukemic effect of the microchimeric maternal cells after infusion into the recipient?

We expect that recent developments in molecular biological tools for microchimerism detection, along with possibilities of isolating microchimeric cells, will propel future research into clarifying functional mechanisms (in)directly associated with the pre-transplant presence of chimeric cells. Viably isolated cells may eventually be cloned and non-specifically increased in numbers using conventional culture conditions. The methodology of HLA-targeted cell sorting may represent an additional tool for phenotypic characterization of microchimeric cells allowing molecular and functional studies of these cells in relation to the host environment. Such studies will step by step unravel the immunobiology behind the epidemiological findings as discussed in this review.

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